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| (54) Title: <b>NEW BIOTECHNOLOGICAL PROCESS FOR PREPARING HYDROXYLATED ML-236B DERIVATIVES, KNOWN AS M-4 AND M-4', AND ANALOGUES THEREOF</b>  |  |   |   |
| (57) Abstract<br><p>The very effective conversion of ML-236B substances and derivatives thereof into 6'-hydroxylyted products with the microorganisms of species <i>Amycolatopsis orientalis</i>, or with an extract or a hydroxylation-effective enzyme derived from said microorganism, is described. The products obtained are suitable as HMG-CoA reductase inhibitors or intermediates thereof. Thus, the products can be used, for example, as an antihypercholesterolemic in pharmacy.</p>   |  |   |   |

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5

Title

10 New Biotechnological Process for Preparing Hydroxylated  
ML-236 B Derivatives, known as M-4 and M-4', and analogues  
thereof

15

Technical Field

The present invention relates to a novel process for  
preparing hydroxylated ML-236 B derivatives, known as M-4  
20 and M-4', and analogues thereof, in particular to an  
enzymatic hydroxylation by means of a microbiological  
process.

25

Prior Art

ML-236B and derivatives thereof as well as analogues are  
known as HMG-CoA reductase inhibitors and are disclosed in  
30 GB Pat. No. 1,555,831. They are produced by fermentation  
with various microorganisms of the genera Gilbertella,  
Streptomyces, Circinella, Monascus, Nocardia, Amycolata,  
Mucor or Penicillium (as disclosed in US Pat. No. 4,346,227  
and US Pat. No. 4,537,859). The hydroxylated forms of  
35 ML-236B, its derivatives and analogues, especially those

known as M-4 and M-4', have been found to be substantially effective inhibitors of HMG-CoA reductase, and their mixture in the form of a pharmaceutically acceptable salt such as the sodium salt has a therapeutic value as an  
5 antihypercholesterolemic agent. All substances exist in the form of an acid or a lactone.

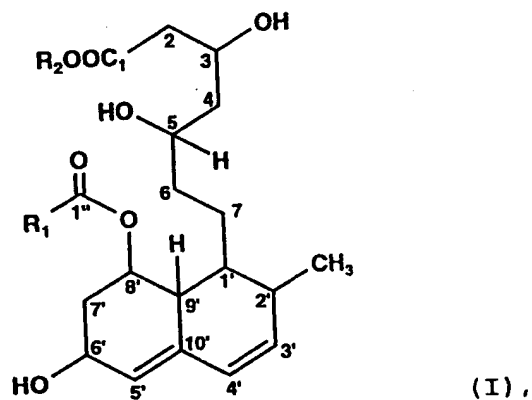
Conventionally, the preparation of pharmaceutically acceptable salts of the HMG-CoA reductase inhibitors, such  
10 as the M-4 and M-4' substances, is a feed-batch process. The first part comprises preparation of a ML-236B substance by fermentation with microorganisms of the genus *Penicillium* and its isolation using conventional isolation techniques and preparation of the sodium salt thereof as described in  
15 US Pat. No. 4,137,322. The second part comprises cultivation of a microorganism of one of the aforementioned genera in the medium to which ML-236B, typically in the form of sodium salt, is added, isolation of the M-4 and M-4' substances and optionally preparation of pharmaceutically acceptable salts  
20 thereof as disclosed in US Pat. No. 4,346,227 and US Pat. No. 4,537,859.

### Object of the Invention

25 There exists a constant need for a novel and improved biotechnological process which will enable the most effective conversion of a ML-236B substance and derivatives and analogues thereof into their hydroxylated forms. The  
30 higher yield in conversion into the hydroxylated forms is also important because of high price of the starting substance.

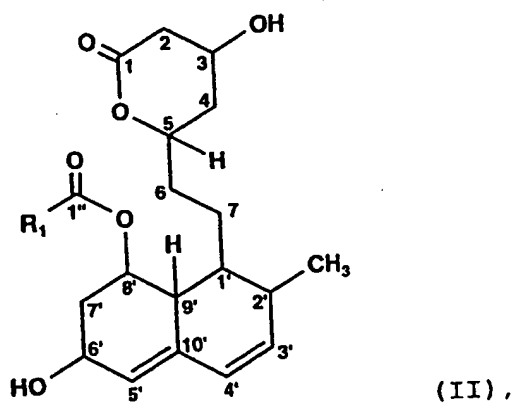
Description of the Invention and the Preferred Embodiments

- 5 The object of the invention has been solved by the provision of a process for preparing a compound defined by the following formula (I)



- wherein  $R_1$  represents substituted or unsubstituted alkyl or substituted or unsubstituted aryl, and  $R_2$  independently represents H, substituted or unsubstituted alkyl or a cation;

or the corresponding lactone (II)



wherein  $R_1$  is as defined above;

which process comprises contacting a 6'-H analogue of the above formula (I) or (II) for hydroxylation with a microorganism of the species *Amycolatopsis orientalis* or with an extract or a hydroxylation-effective enzyme derived  
5 from said microorganism.

According to the present invention a specific microorganism was found which, being used in a microbiological process or in the form of an extract or a hydroxylation-effective  
10 enzyme derived from said microorganism, converts desired compound substrates, namely the 6'-H analogue of the compounds of formula (I) or the lactone of formula (II), into their hydroxylated forms with improved, relatively high yields. Various microorganisms reported in the literature as  
15 potential producers of the hydroxylated forms have been studied, and it has surprisingly been found that the best results are obtained when employing the microorganism of *Amycolatopsis orientalis* and especially when employing the strain ATCC 19795, or the extract or the hydroxylation-  
20 effective enzyme derived therefrom. *Amycolatopsis orientalis* is to date merely known as a producer of antibiotic vancomycin.

The strain *Amycolatopsis orientalis* ATCC 19795 is  
25 characterized by a well-branched mycelium which under specified conditions fragments into shorter particles. Colonies grown on a solid agar culture medium are of a pale blue colour, velvet-like appearance, slightly folded and upheaved, 5 to 10 mm in diameter. The most convenient  
30 temperature for growth is within the range from 24 °C to 30 °C, with a notion that this microorganism poorly sporulates.

The subject of the present invention is the enzymatic  
35 hydroxylation process which can be suitably effected by contacting the aforementioned 6'-H analogue of the compound

of formula (I) or the corresponding lactone (II) with the microorganism *Amycolatopsis orientalis*, especially the strain deposited as ATCC 19795, in a suitable fermentation medium.

5

A fermentation medium should preferably comprise a source of assimilable carbon, such as glucose, saccharose, dextrans, glycerol, starch, soybean oil and molasses, a source of assimilable nitrogen, such as soybean flour, meat and yeast  
10 extracts, peptones, ammonium salts and, if required, various inorganic salts, such as sodium chloride, potassium chloride, magnesium sulphate, calcium carbonate and phosphates. Most conventional culture media may be used that employ microorganisms of the genus *Amycolatopsis* for  
15 fermentation. Suitable inoculation and fermentation media are, for example, described by J.J. McIntyre et al. in *Biotechnology and Bioengineering*, vol. 49, pp. 412-420 (1996); L.D. Boeck et al. in *The Journal of Antibiotics*, Vol. XXXVII No. 5, pp. 446-453 (1984); G.J. Clark et al. in  
20 *Microbiology*, Vol. 141, Pt. 3, pp. 663-669 (1995); and in US Patent No. 4,547,488; all descriptions being incorporated herein by way of reference.

Fermentation is carried out under aerobic conditions and at  
25 a temperature within a suitable range, for example from 20° to 36 °C, preferably from 24 °C to 30 °C. The aforementioned substrate compound to be hydroxylated may be contacted with the microorganism, preferably in the form of sodium salt, at any time in the course of fermentation or after completion  
30 of the fermentation, but an addition at the vegetative part of fermentation, which is normally between 24 and 48 hours after beginning cultivation, is particularly preferred. The compound substrate is added to a total final concentration of 0.01 wt.-% to 5 wt.-%, preferably 0.05 wt.-% to 0.5  
35 wt.-%, based on the total fermentation broth weight or the contacting liquid. Addition to a fermentation medium may be

either batch, feed-batch or continuous. Fermentation is usually completed within 2 to 5 days after addition of the substrate compound. Then, the microorganism cells are separated, preferably by filtration, and the resulting filtrate is extracted with an organic solvent which is preferably water immiscible or has a limited miscibility with water, such as ethyl acetate, ether (for example diethyl ether) or chloroform. Subsequently, the organic solvent is removed, suitably by evaporation, and if desired the resulting crude product is subjected to further conventional purification and isolation processes, for example employing column chromatography, e.g. using silica gel column, and eluting the desired compound with an appropriate eluent. If required, the resulting product may be subjected to recrystallization, salification (if it is desired in the form of salt), lactonization or esterification, with methods known to those skilled in the art.

If the contacting step is carried out after completion of the fermentation such as about 4 to 5 days after beginning the cultivation, the microorganism cells can be collected, for example by filtration, optionally washed with an appropriate buffer solution such as phosphate buffer, and then contacted with the substrate compound in an appropriate buffer solution (pH from 5 to 9 at a temperature suitably from 20 to 45 °C, preferably from 25 to 30 °C).

Alternatively, the hydroxylation process can also be effected by a cell-extract of *Amycolatopsis orientalis*. The cell-extract may be used as it is, or it may be used in a modified form, e.g. previously drying it or separating non-hydroxylation effective cell constituents. Furthermore, an hydroxylation-effective enzyme derived from said microorganism can be employed for the hydroxylation process. For example, an enzyme being effective for the hydroxylation step is previously isolated from *Amycolatopsis orientalis*.



Also contemplated within the concept of employing an enzyme derived from the microorganism is the use of a modified enzyme, for example a stabilized enzyme or an enzyme being bound to a support, and the use of a genetically engineered enzyme corresponding to the hydroxylation-effective enzyme derived from said microorganism. Suitable methods for obtaining the cell-extract or hydroxylation-effective enzyme, or their modified forms, are well known to those skilled in the art.

10

The product obtained by the microbiological process is typically a mixture of both 6'-hydroxy  $\alpha$ - and  $\beta$ -configurations and can be used as such. If desired, the isomers with the respective  $\alpha$ - and  $\beta$ -configuration may be separated by an appropriate isomer resolution technique known to those skilled in the art.

15

The structural moieties of  $R_1$  and  $R_2$  can be selected from the definitions as specified above for obtaining HMG-CoA reductase inhibitors or intermediates thereof.

Thus,  $R_1$  may represent optionally substituted alkyl or optionally substituted aryl. The alkyl group include straight chain, branched or cyclic hydrocarbon optionally having unsaturated double bonds and optionally being substituted. The main chain of the alkyl group has 1 to 15, preferably 1 to 10 and more preferably 1 to 6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, hexyl, the isomers and the branched derivatives thereof, and the like. The optional substituents include one or more of the group consisting of halogen such as chloro, amino, lower alkyl amino such as mono- or dimethylamino, hydroxy, alkoxy, cyano, nitro, and the like. A preferred substituent is hydroxy. If present, a branching hydrocarbon group preferably has 1 to 4 carbon atoms, such as methyl and ethyl. Possible aryl residues include substituted or unsubstituted phenyl, biphenyl and naphthyl,

20

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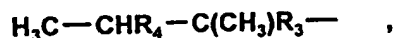
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35

wherein the optional substituents are selected from the group consisting of lower alkyl, alkoxy, halogen such as chloro, amino, lower alkyl amino such as mono- or dimethylamino, hydroxy, alkoxy, cyano, nitro, and the like.

5

It is particularly preferred that  $R_1$  represents an alkyl residue having the following structure:



wherein  $R_3$  denotes H or  $CH_3$  and  $R_4$  independently denotes H or OH.

10

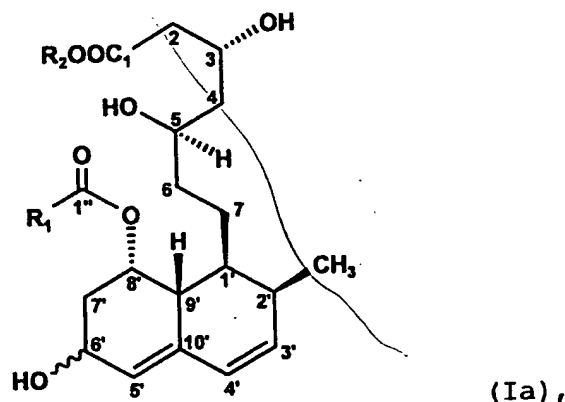
Furthermore,  $R_2$  may represent H, alkyl or a cation, corresponding to the presence of a free acid, an ester or a salt of the compound of formula (I), respectively. Possible alkyl residues include straight chain, branched or cyclic hydrocarbon optionally having unsaturated double bonds and optionally being substituted, as previously described with respect to  $R_1$ . Possible cations include metal cations, for example sodium, potassium, lithium, calcium, magnesium, preferably alkali metal cations such as sodium, an ammonium group, an alkyl ammonium group, and the like.

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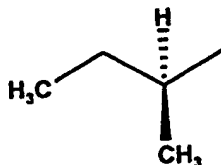
In order to provide a more potent HMG-CoA reductase inhibitor, the prepared compound of formula (I) has the following configuration (Ia):

25

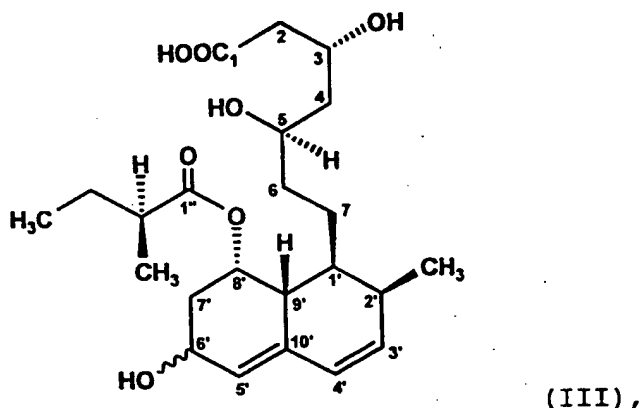


or the corresponding lactone compound (IIa);  
 wherein  $R_1$  and  $R_2$  are as defined above, and the  $6' \sim OH$ -  
 group represents an  $\alpha$ - or a  $\beta$ -configuration, or a mixture of  
 5 both  $\alpha$ - and  $\beta$ -configurations. The compounds of formula (Ia)  
 or (IIa) are obtained by starting with a  $6'-H$  analogue  
 having the corresponding configuration.

A particularly preferred example for  $R_1$  is an alkyl residue  
 10 having the following structure and configuration:



In a specifically preferred embodiment of the present  
 invention, a compound having the following formula (III) or  
 15 the salt thereof or the corresponding lactone is prepared in  
 order to provide a particularly active and potent HMG-CoA  
 reductase inhibitor or an intermediate thereof:



wherein the 6'  $\sim$ OH-group represents an  $\alpha$ - or a  $\beta$ -configuration, or a mixture of both  $\alpha$ - and  $\beta$ -configurations.

- 5 If the salt is prepared, it is typically the pharmaceutically acceptable salt, in particular the sodium salt.

For obtaining the starting compounds for the process of the present invention, and for preparing the derivatives, analogues and variations (with respect to the definitions of  $R_1$  and  $R_2$ ) such as the lactone form, the ester form or the salt form from the obtained 6'-hydroxylated product, reference is made to the descriptions of GB Patent No. 1,555,831, US Pat. No. 4,346,227 and US Pat. No. 4,537,859.

15

The compounds obtained by the process according to the present invention can be further modified by synthetic, semi-synthetic or biochemical procedures. Thus, the compounds obtained in the present invention may themselves constitute intermediates for producing further HMG-CoA reductase inhibitors. For example, one or both double bonds in the ring structure of the obtained compounds of formula (I) or (II) may be hydrogenated.

20

- 25 The HMG-CoA reductase inhibitors obtained by the process according to the present invention, which are preferably purified to at least 99.5%, more preferably 99.6 %, can be beneficially used for the preparation of a pharmaceutical

for the prevention and/or treatment of diseases. The compounds obtained by the process according to the present invention, or the HMG-CoA reductase inhibitors derived therefrom, can be effectively used as an antihyper-  
5 cholesterolemic agent. The compounds can therefore be used for the preparation of a medicament for the control of cholesterol in the body of an individual. They can further be used for the prevention or the treatment of atherosclerosis. The obtained inhibitors and pharmaceuticals are  
10 particularly useful as preventives for reducing the risk of stroke, transient ischemic attack and myocardial infarction.

This invention is illustrated but in no way limited by the following examples.

15

#### Example 1

##### *Preparation of the inoculum*

20 The rationale for determining the most suitable productive culture was selection of individual colonies with the typical morphological characteristics. The selected colonies were transferred to a sterile potter to agar slopes and homogenised. The resulting colonies were transferred and  
25 incubated in the thermostat at 26 °C to 30 °C for 7 to 14 days. During that time surfaces of agar slopes were overgrown by a culture of homogeneous, folded, smooth, white to pale greyish-blue mycelium. A portion (0.5 to 1 ml) of the resulting culture was then inoculated into a vegetative  
30 medium.

35

Agar medium for preparation of agar slopes and petri plates:

| Raw material       | Amount        |
|--------------------|---------------|
| Dextrin            | 10 g          |
| Consumer's glucose | 5 g           |
| Casaminic acid     | 3 g           |
| Yeast extract      | 4 g           |
| CaCO <sub>3</sub>  | 1 g           |
| Agar               | 15 g          |
| Sterile water      | up to 1000 ml |

No pH adjustment needed.

5

#### *Vegetative phase of fermentation*

- 10 The inoculum grown on agar slope at 26 °C to 30 °C for 10 days and prepared according to the method described in Example 1, was inoculated on a 500-ml Erlenmeyer flask containing 50 ml of the vegetative medium. After 2 days a portion of the culture (5 to 10%) was transferred onto the
- 15 fermentation medium.

#### *Vegetative medium*

| Raw material  | Amount |
|---|--------|
| Corn starch for fermentation                          | 20 g   |
| Soybean flour for fermentation                        | 14 g   |
| Glucose   | 3 g    |
| Yeast extract   | 5 g    |
| NaH <sub>2</sub> PO <sub>4</sub> x 2 H <sub>2</sub> O | 3.3 g  |

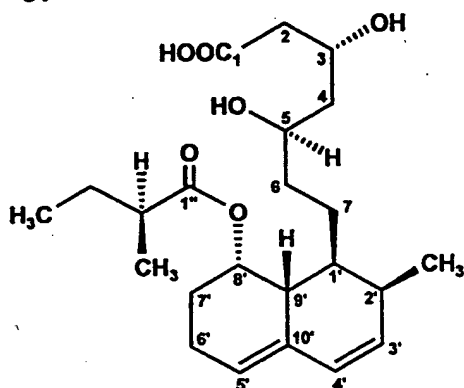
Tap water to 1000 ml

20

**Example 2**

Conversion of a ML-236B substance into 6'-hydroxy derivatives M-4 and M-4' thereof

- 5 A portion of the culture, prepared according to the method described in Example 1, was transferred into Erlenmeyer flasks with fermentation medium 1 to which a ML-236B substance having the formula shown below, in the form of sodium salt, was added to a concentration of 200 mg/L on the
- 10 second day of fermentation. Analyses of the concentration of the 6'-hydroxylated M-4 and M-4' compounds in the fermentation broth showed the total final concentration of the M-4 and M-4' substances in the fermentation broth to be
- 15 60 mg/ml after 3 days of fermentation at temperature between 24 °C and 30 °C.



Fermentation medium 1

20

| Raw material                            | Amount |
|---|--------|
| Glycerol                                | 80 g   |
| Soybean flour                           | 20 g   |
| Soybean oil                             | 0.1 g  |
| Calcium gluconate                       | 12 g   |
| Magnesium chloride x 6 H <sub>2</sub> O | 1.3 g  |
| Magnesium sulphate x 7 H <sub>2</sub> O | 1 g    |

|                   |      |
|-------------------|------|
| NaNO <sub>3</sub> | 10 g |
|-------------------|------|

Tap water to 1000 ml

- All raw materials were dissolved in tap water, the pH was then adjusted to a value of 7.8 by addition of 1M aqueous solution of NaOH. The resulting medium was poured into 300 ml-Erlenmeyer flasks, 30 ml per flask.

### Example 3

- 10 *Conversion of a ML-2368 substance into 6'-hydroxy derivatives M-4 and M-4' thereof*

- A portion of the culture, prepared according to the method described in Example 1, was transferred into Erlenmeyer flasks with fermentation medium 3 to which ML-236B, in the form of sodium salt, was added to a concentration of 400 mg/L on the second day of fermentation. Analyses of the concentration of M-4 and M-4' substances in the fermentation broth showed the total final concentration of the M-4 and M-4' substances in the fermentation broth to be 160 mg/ml after 3 days of fermentation at temperature between 24 °C and 30 °C, indicating a 40% conversion.

- 25 Fermentation medium 3

| Raw material                            | Amount |
|---|--------|
| Glycerol                                | 20 g   |
| Soybean flour                           | 20 g   |
| Calcium gluconate                       | 12 g   |
| Magnesium chloride x 6 H <sub>2</sub> O | 1.3 g  |
| Magnesium sulphate x 7 H <sub>2</sub> O | 1 g    |
| NaNO <sub>3</sub>                       | 10 g   |
| Tap water to 1000 ml                    |        |



All raw materials were dissolved in tap water, the pH was then adjusted to a value of 7.8. The resulting medium was poured into 300 ml-Erlenmeyer flasks, 30 ml per flask.

5

**Example 4**

*Conversion of a ML-236B substance into 6'-hydroxy derivatives M-4 and M-4' thereof at pilot plant scale*

- 10 The contents of ten Erlenmeyer flasks with the culture, prepared according to the method described in Example 1, whereat the vegetative part of fermentation was shorten to 24 hours, were used to inoculate the fermenter (14 l) with 10 l of fermentation medium. After 24-hour fermentation,
- 15 ML-236B, in the form of sodium salt (470 mg of ML-236B dissolved in 500 ml of water) was continually added to the medium for 6 hours. Analyses of the concentration of M-4 and M-4' substances in the fermentation broth showed the total final concentration of the M-4 and M-4' substances in the
- 20 fermentation broth to be 300 mg after 36 hours of fermentation at temperature between 24 °C and 30 °C, indicating a 64% conversion.

**Fermentation medium 2**

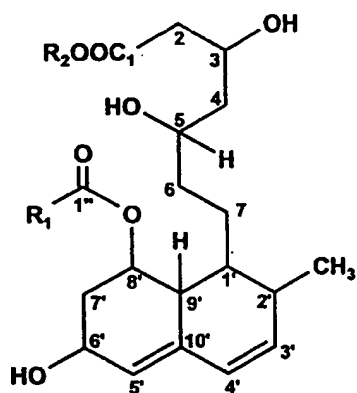
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| <b>Raw material</b>                                   | <b>Amount</b> |
|---|---------------|
| Glycerol  | 20 g          |
| Corn starch for fermentation                          | 20 g          |
| Soybean flour for fermentation                        | 14 g          |
| Glucose   | 10 g          |
| Yeast extract   | 5 g           |
| NaH <sub>2</sub> PO <sub>4</sub> x 2 H <sub>2</sub> O | 3.3 g         |
| Tap water to 1000 ml                                  |               |

All raw materials were dissolved in tap water, the pH was then adjusted to a value of 7.8.

## Claims

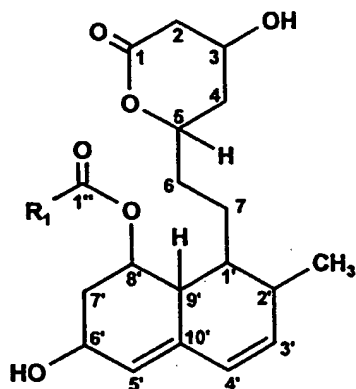
- 5 1. A process for preparing a compound defined by the following formula (I)



(I),

- wherein  $R_1$  represents substituted or unsubstituted alkyl or substituted or unsubstituted aryl, and  $R_2$  independently  
 10 represents H, substituted or unsubstituted alkyl or a cation;

or the corresponding lactone (II)

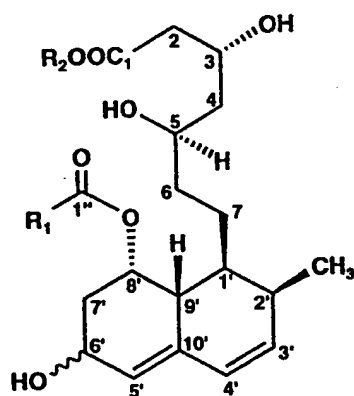


(II),

- 15 wherein  $R_1$  is as defined above;

which process comprises contacting a 6'-H analogue of the above formula (I) or (II) for hydroxylation with a microorganism of the species *Amycolatopsis orientalis* or with an extract or a hydroxylation-effective enzyme derived from said microorganism.

2. A process according to claim 1, wherein the prepared compound of formula (I) has the following configuration (Ia):

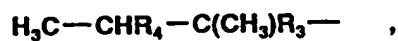


(Ia).

or the corresponding lactone compound (IIa); wherein  $R_1$  and  $R_2$  are as defined in claim 1, the 6'  $\sim$ OH-group represents an  $\alpha$ - or a  $\beta$ -configuration, or a mixture of both  $\alpha$ - and  $\beta$ -configurations.

3. A process according to claim 1 or 2, wherein  $R_2$  represents H, a  $C_1$ - $C_6$  alkyl group or a cation selected from the group consisting of an alkali metal, an ammonium group or an alkyl ammonium group.

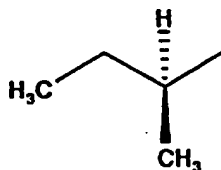
4. A process according to any one of claims 1 to 3, wherein  $R_1$  represents an alkyl residue having the following structure:



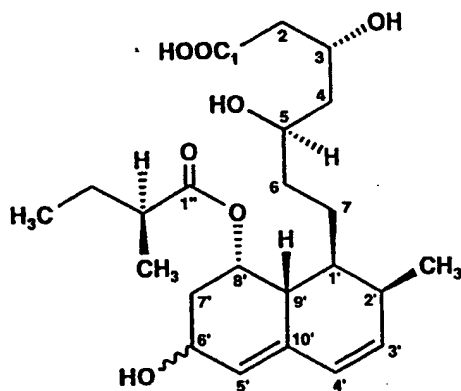
wherein  $\text{R}_3$  denotes H or  $\text{CH}_3$  and  $\text{R}_4$  independently denotes H or OH.

5

5. A process according to claim 4, wherein said alkyl residue has the following structure and configuration:



6. A process according to claim 1, wherein a compound  
10 having the following formula (III) or the salt thereof or the corresponding lactone is prepared:



(III),

15

wherein the  $6' \sim \text{OH}$ -group represents an  $\alpha$ - or a  $\beta$ -configuration, or a mixture of both  $\alpha$ - and  $\beta$ -configurations.

20

7. A process according to any one of the preceding claims, wherein said microorganism is *Amycolatopsis orientalis* ATCC 19795.

5 8. A process according to any one of the preceding claims, wherein said contacting step is performed in the course of fermentation of said microorganism, and wherein said 6'-H analogue is added to the fermentation broth in a batch, feed-batch or continuous manner.

10

9. A process according to claim 8, wherein said 6'-H analogue is added to the fermentation medium to the final content of 0,05 to 0,5 wt.-%, based on the total medium weight.

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10. A process according to claim 8, wherein sources of assimilable carbon, nitrogen and/or phosphorus is/are added during the fermentation.

20

11. A process according to any one of the preceding claims which is used for the preparation of a HMG-CoA reductase inhibitor.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/00923

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12P7/62 C12P7/42

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

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Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International Application No

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| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
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